

The University of Chicago

**STUDIES ON THE METABOLISM OF
SARCINA LUTEA**

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

1933

By

BORIS BENJAMIN RUBENSTEIN

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DECREASE IN RATE OF OXYGEN CONSUMPTION
UNDER THE INFLUENCE
OF VISIBLE LIGHT ON
SARCINA LUTEA

For these experiments cultures of the micrococcus *S. lutea* were grown for twenty-four hours on 1 per cent. peptone agar. The organisms were then gently washed from the medium with distilled water, centrifuged rapidly, washed again and finally suspended in such a quantity of distilled water that there were approximately 5×10^8 cells in each cubic centimeter. Of this suspension, one cubic centimeter was pipetted into each of four reaction vessels of Warburg manometers;¹ one cubic centimeter was heated to boiling for a minute, made up to volume and pipetted into a fifth reaction vessel; the remainder of the suspension was used to inoculate new cultures and for other purposes. The reaction chamber of a sixth manometer contained one cubic centimeter of sterile distilled water. These vessels, into the side-arm of which a half cubic centimeter of normal base (NaOH) had been introduced, were then affixed to their respective manometers. The manometers were set in position in the water bath and allowed to shake for half an hour with open stopcocks, until temperature equilibrium. Then the stopcocks were shut; and since the base absorbed all the carbon dioxide excreted, the change in pressure registered by the manometers can be attributed wholly to the consumption of oxygen by the *Sarcina* in the closed system.

¹ O. Warburg, "Über d. Stoffwechsel d. Tumoren," 1926.

The water bath was thermostatically controlled so that the temperature did not vary more than $\pm 0.02^\circ \text{C.}$, during the course of any experiment. Illumination was provided by 10 60-w. frosted Mazda lamps arranged in two rows below the water bath, the light

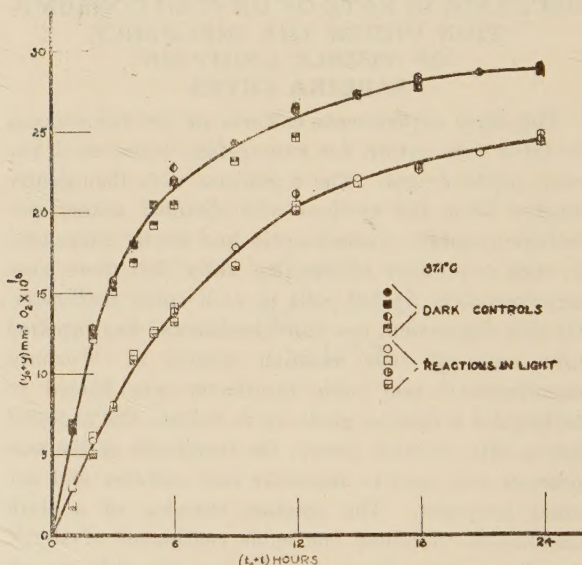


FIG. 1. Curves plotted to data from four series of experiments in distilled water and inorganic salts. Each point represents the arithmetic mean of twenty determinations. t_0 is the time elapsed between the beginning of starvation and the beginning of measurement; similarly, y_0 is the oxygen consumption during the period t_0 . Both t_0 and y_0 are presented as unknown.

passing through the plate glass bottom of the tank and then nine inches of water before impinging on the reaction vessel. The two vessels in which we measured the oxygen consumption in the dark were wrapped in tin foil. The intensity of illumination

was frequently controlled and found to be 556 ± 8 meter candles at the level of the vessels.

Fig. 1 shows a pair of curves drawn through the plotted data of four pairs of series, each plotted point representing the arithmetic mean of twenty observations. In from 48 to 72 hours, the oxygen consumption falls off to practically nil, while the total quantity of oxygen consumed is approximately equal in the irradiated series and in the dark controls. At the end of a run, the *Sarcina* were plated out to make a count of viable organisms; the results of the counts indicate either that there had occurred no appreciable number of deaths during the period, or that the number of deaths was compensated by the appearance of an almost equal number of new organisms.

The gradual falling off in the rate of oxygen consumption is of interest, especially since we could attribute it neither to a limitation of oxygen supply nor to an accumulation of metabolites in the suspension medium. Apparently, the only substance excreted into the medium by the cells is carbonic acid; since we could find in it no traces even of fatty acids or of nitrogenous compounds. And, furthermore, if we added small quantities of fatty acids or alcohols, we found either that they did not affect the rate of oxygen consumption and were left in the suspension medium, or that they materially accelerated the rate of oxygen consumption and were taken up by the cells from the medium.

It may be noted that of the four series of data plotted, one describes the oxygen consumption of *S. lutea* in distilled water, one in phosphate buffer² (pH 6.5-8.0), one in half normal sodium chloride solution, and the fourth in tenth per cent. potassium

² M. Clark, "The Determination of Hydrogen Ion."

cyanide solution. We could as well have plotted the data taken on the oxygen consumption of *S. lutea* in a medium of normal potassium iodide, tenth normal potassium chloride, fiftieth molar ferric chloride, or Ringer solution. The data all lie scattered along the same curves; the effects of the different ions and of the different osmotic pressures being less than the experimental error for any determination, that is, less than 3 per cent. of the mean value of twenty determinations in any other of the suspension media. Similar lack of effect of changing osmotic pressure has been reported by Falk,³ and is discussed by Beck.⁴ Thus, it appears that *S. lutea* is an organism in which the respiration is not responsive to changes in the osmotic pressure of its suspension medium; and furthermore, in which iron catalysis of respiration does not occur⁵—or if it does occur, normally, is not essential for the maintenance of cell oxidations.

By inspection, it is evident that even though the total oxygen consumption turns out to be equal in the two series, the rates of oxygen consumption diverge somewhat widely when the cells are irradiated in one case and in the other when they are shielded from light. Indeed, during the first hour, while there is apparently a quantity of light-sensitive material in the cells, the rate for the dark control (at this temperature—37.1° C.) may be as much as five times that for the irradiated organisms.

I wish to acknowledge the invaluable assistance of Dr. R. S. Lillie and Dr. R. W. Gerard, of this department.

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³ I. S. Falk, *Abstracts Bact.*, 7: 87–103, 133–147, 1923.

⁴ W. A. Beck, *Plant Physiol.*, 3: 413–440, 1928.

⁵ O. Warburg, *Biochem. Zeit.*, 136: 266–77, 1923.

THE KINETICS OF INTRACELLULAR CARBOHYDRATE OXIDATION OF *SARCINA LUTEA*

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SIX FIGURES

(Received for publication May 31, 1932)

It has been shown that the rate of oxygen consumption of *S. lutea* in sugar solutions falls off continuously with time (6, 11). This datum was so suggestive that it seemed interesting to attempt to determine the chemical kinetics of an intracellular oxidation reaction, despite the fact that the enterprise might appear foolhardy at a time when the course of so few reactions, except in gases, has been established.

Whatever the scheme postulated for the oxidation of carbohydrate, we may safely assume that there must be a chain of reactions, some of which are monomolecular in the presence of excess water. It was found, in fact, that the rate of oxygen consumption in dextrose solution follows a course corresponding to the requirements of the monomolecular formula. The general monomolecular equation in the differential form is

$$(1) \quad \frac{dx}{dt} = k(a - x)$$

where x is the quantity of substrate converted, a is the original quantity of substrate, t is the time, and k is the velocity constant typical for the reaction. Since we measured neither the original amount of substrate nor the quantity oxidized, it was necessary to make the reasonable assumption that each increment of oxygen consumption is equivalent to an increment of substrate oxidized, or

$$(2) \quad y = bx$$

where y is the oxygen consumed, and b is a constant of proportionality. From this it follows that the original quantity of substrate, a , can be set proportional to the maximum oxygen consumption when the reaction has run to completion, or

$$(3) \quad A = ba$$

where A is the maximum attainable oxygen consumption. With these new terms, the original equation 1 becomes

$$(4) \quad \frac{dy}{dt} = \frac{k}{b} (A - y) = K (A - y)$$

These simple transformations enable us, with the data at hand, to determine whether the equation describes the course

TABLE 1
Direct solution for K in the equation: $\frac{dy}{dt} = K (a - y)$

DURATION OF EXPERIMENT, HOURS	CONCENTRATION OF GLUCOSE, PER CENT	TEMPERATURE, °C.	K × 100		REMARKS
			Irradiated	Dark	
6	.01	37.2	38	42	Irradiated reaction was not complete in 6 hours
12	.025	37.1	38	42	
24	.05	37.1	37	41	
24	.10	37.2	37	42	
24	.50	37.1	38	41	At end of 24 hours glucose still present
48	1.00	37.1	38	41	
48	.50	20.0	12	10	
48	2.00	20.1	13	10	

of the reaction in *S. lutea*. This may be done by direct algebraic solution of the equations for K , after setting $A = 100$ per cent, or unity, as is shown in table 1; or by plotting the data $\frac{dy}{dt}$ against y and fitting straight lines to the plot by the method of least squares (8), as is shown in figure 1. Both methods show reasonably concordant values for K .

Instead of the differential, the integral form of equation 4 may be used.

$$(5) \quad y = A (1 - e^{-Kt})$$

Again we may solve directly or fit by the method of least squares. Since an error in t , the time measurement, is of less significance using this form of the equation, better results

are to be expected than with the differential form. Figure 2, indeed, shows this to be true.

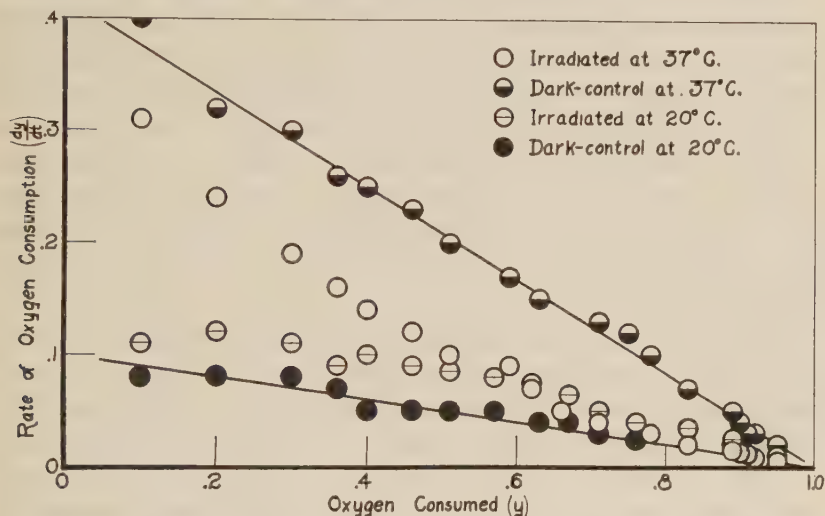


Fig. 1 The lines were drawn to calculated values of K rather than through the plotted points which represent successive increments of oxygen consumption. Successive increments are apt to be 'step-like' in character, and so would tend to elevate the apparent value of K . No single line could fairly be drawn through the values for oxygen consumption during irradiation.

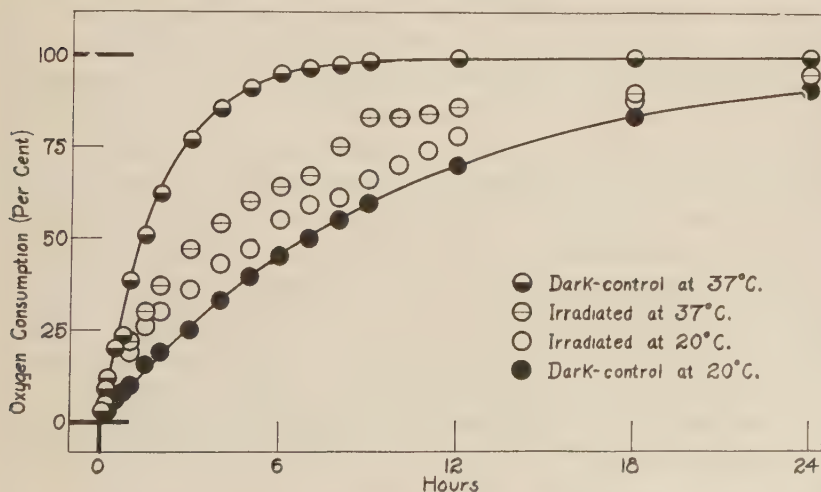


Fig. 2 The curves for the irradiated reaction would fall between these two curves. The lines are drawn through calculated values of K rather than through the observed values, which are plotted.

EXPERIMENTAL DETAIL

The experimental procedure was essentially the same as that described in a previous paper(11), with one important modification. Before any measurements were taken, the cells, removed from the nutrient agar slants on which they had grown, were washed in distilled water and resuspended in neutral, M/15, Sørensen phosphate buffer(2). Air was then bubbled slowly through the suspension overnight, the suspension being kept at room temperature, but in the dark. In the morning, the cells were centrifuged down. The supernatant buffer was decanted. Then the cells were resuspended in the desired menstruum. Two cubic centimeters of the cell suspension were placed in each of the Warburg micro-respirometer vessels. Into one side arm of each vessel was placed 0.5 cc. of substrate, usually glucose, of such concentration that the final concentration of glucose (after tipping) would be as recorded. Into the other side arm was put 0.5 cc. of normal sodium hydroxide. The vessels were attached to their manometers and permitted to come to temperature equilibrium before measurements were begun. This usually required about thirty minutes.

Several measurements were taken before the nutrient substrate was tipped from the side arm of the vessels into the cell suspension. Thus we assured ourselves that 'endogenous' cell respiration had become negligible (less than 1 cu.mm. per hour) before we measured the oxygen consumption induced by the addition of substrate. Consequently, we were able to begin measuring oxygen consumption from the moment the substrate was added.

Cell counts were made before and after each experiment, 1) with a hemocytometer and, 2) by streaking on nutrient agar plates, incubating and counting colonies. We noted that during the course of our experimental procedure there was no significant change in the number of cells present in the suspension, 2 cc. of which held approximately 10^{10} cells.

DISCUSSION

From the charts presented, it can be seen at once that the oxygen consumption of *S. lutea* in the dark, but not in the light, can be described by the equation for the monomolecular process. It seems obvious, if any proposed scheme of oxidative breakdown of carbohydrate be accepted, that in our work only one member of a concatenated series of reactions has been measured, and that one the slowest, or 'master,' reaction. Just which reaction this is should be discovered either, 1) by using suitable inhibitors for the different stages or, 2) by supplying each of the proposed intermediate products and noting the stage at which the previously found constants disappear. The inhibitor technique presents the unusual difficulty that the ordinary inhibitors, viz., cyanide or carbon monoxide, fail to inhibit more than a small fraction of the oxygen consumption of *S. lutea*, as has been shown by Gerard(7). At present we are attempting the second of these methods.

Again, if the process is monomolecular, then the rate of oxygen consumption should be proportional to the amount of substrate added. No direct measure for carbohydrate concentration within the cell is available. Yet, if the carbohydrate concentration at the site of oxidation is proportional to the carbohydrate concentration in the suspension medium, then proportionality between rate of oxygen consumption and external carbohydrate concentration may be considered as further confirmatory evidence for the hypothesis of a monomolecular process. Figure 3 shows that true proportionality exists in the range 0.01 M glucose to 0.125 M glucose. At higher concentrations, the proportionality factor slowly decreases. At 3.5 M glucose, there is some evidence of toxic injury, viz., reduction in the plate count of colonies grown from viable organisms. This is probably osmotic injury, since a similar injurious effect is noted with 2.2 N NaCl.

The oxygen-consumption results during irradiation do not fit the theoretical curves accurately. We must be measuring either an entirely different process, or perhaps the sum of two processes. Since the dark reaction has been determined,

the latter hypothesis seems to be the simpler and the more reasonable. During irradiation two concurrent processes involving oxygen consumption may be assumed, one apparently monomolecular and the other possibly photochemical. This latter reaction seems unimportant at the higher temperature (37°), but becomes relatively more important as the temperature falls. This we should expect, since most photochemical reactions have a low temperature coefficient.

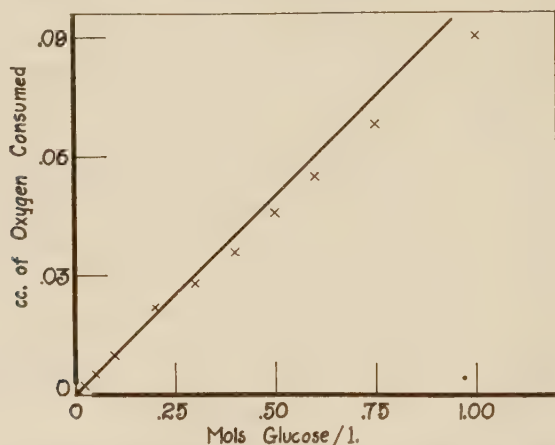


Fig. 3 The oxygen consumption was taken for the first-hour period. The points fall below the straight line at values above 0.25 M. Extension of the curve would show a downward swing beginning at about 3.0 M.

It will be noted (table 1 and figs. 1, 2) that at the higher temperature (37°) there is a decrease in the rate of oxygen consumption during irradiation, particularly with low concentration of glucose (M/100). This confirms our earlier findings(11). On the other hand, at the lower temperatures (20°), and in the presence of relatively high concentrations of glucose, irradiation increases the rate of oxygen consumption (table 2). This behavior suggests the hypothesis that in some way the photochemical reaction diverts oxidizable substrate at high temperatures, while making it more readily available at lower temperatures.

Just what the photochemical reaction is we do not know. It seems likely, however, that some rather stable intermediate compound, perhaps a peroxide, as suggested by Tappeiner(12), is formed. If the photochemical compound (e.g., peroxide) is oxidized in the light less rapidly than the un-

TABLE 2

A typical series of experiments showing the effect of temperature on the rate of oxygen consumption of S. lutea

EXPERIMENT NO.	TEMPERATURE, °C.	CUBIC CENTIMETER OF OXYGEN PER HOUR			
		$\frac{dO_2}{dt}$, normal Sarcinae		$\frac{dO_2}{dt}$, heat-treated Sarcinae	
		Dark	Irradiated	Dark	Irradiated
16	46.4	.30	.30	.30	.30
17	38.9	.42	.40	.26	.25
109	37.1	.44	.37		
18	36.0	.37	.36	.18	.18
126	34.5	.32	.32		
19	32.8	.25	.26	.10	.11
20	30.7	.24	.25	.04	.05
21	28.9	.20	.24	.01	.01
22	27.0	.17	.22	.003	.004
23	24.1	.13	.19	.001	.001
24	21.8	.11	.17	1/∞	1/∞
25	19.7	.10	.15		
26	18.6	.10	.13		
28	17.5	.08	.12		
31	16.8	.08	.12		
32	12.3	.07	.10		
33	8.5	.03	.06		
35	5.1	.02	.05		
36	4.0	.01	.03		

NOTE: In this series of experiments, the glucose concentration was M/5. When lower concentrations of glucose are used, the decrease in rate of oxygen consumption at 37° during irradiation is much more marked.

altered substrate at the higher temperature, and more rapidly than the unaltered substrate at the lower temperature, the observed effects would be accounted for. This would imply that at some intermediate temperature oxygen consumption would be the same in the light and in the dark. This, indeed, we have observed, as can be seen from the intersection of the curves in figure 4, at approximately 30°C. Thus, when there

is a low concentration of glucose, the formation of intermediary stable peroxides may actually prevent a more rapid direct oxidation. While, when the conditions are so altered that the direct oxidation is decreased, as by reducing the temperature, then the photochemical reaction may be adjuvant.

There is yet another approach to the problem. It has been the experience of chemists that a study of changes of reaction velocity that occur with changes of temperature may give

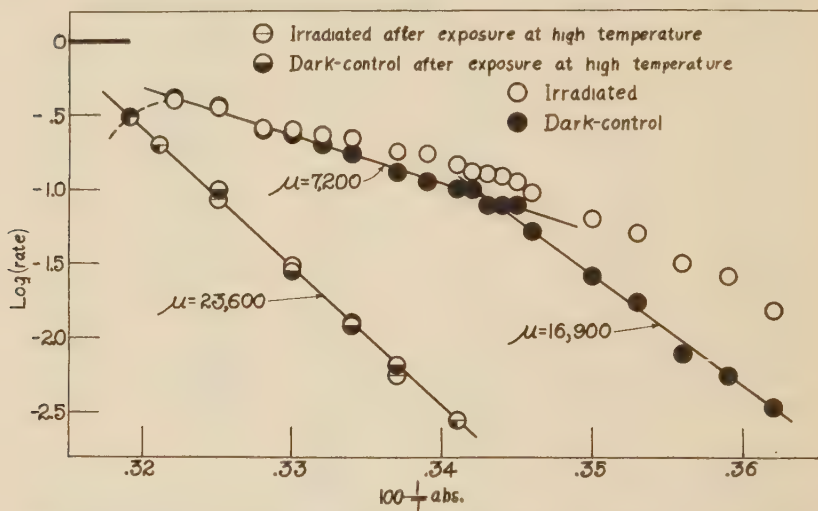


Fig. 4 No curve was drawn through the series of values obtained during irradiation, since there is no true constant velocity during irradiation and since many curves might be drawn with equal fairness.

significant results. That the Q_{10} ratio was a variable quantity in even a single reaction between gases proved disturbing to Van't Hoff, who proposed an equation which would describe more accurately the actual changes in velocity occurring with changes in temperature(13). A theoretical basis for this equation was supplied by Arrhenius(1), who assumed that the changes in rate might be due to changes in the 'heat of activation' required by different catalytic systems. The equation is

$$(6) \quad \frac{V_1}{V_2} = e^{\frac{\mu}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right)}$$

where V_1 and V_2 are the reaction velocities at absolute temperatures T_1 and T_2 , respectively; e is the base of the natural logarithm system; R is the universal gas constant; and μ is a constant with the dimension of calories and is called the 'critical thermal increment' by Crozier(3).

Since, in simple reactions, the reaction velocity is easy to determine, particularly in monomolecular reactions where the reaction velocity is identical with the velocity constant of the reaction, K ; since R in the c.g.s. system is approximately 2; and since T is measured; there is only one unknown in the equation, μ .

To facilitate solution of the equation, we may take logarithms. The equation then becomes—

$$(7) \quad \ln \frac{V_1}{V_2} = -\frac{\mu}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

This may be solved directly, or by plotting $\ln V$ against $\frac{1}{T}$, in which case μ is the slope of the resulting curve. The graphic method is to be preferred, since it provides both a summary of the data and a solution, as is shown in figure 4.

The experimental technique has already been described. In the present series of experiments glucose was supplied in one concentration, 0.1 M, and the temperature was varied by small intervals from 4°C. to 44°C.

Before drawing any conclusions from our data, it may be well to summarize the present status of this type of analysis. In 1924, Crozier reviewed the subject of the significance of critical thermal increments in biology(3). He had found, in analyzing the data of many workers, that for respiratory processes three values are most frequently found for μ : 11,500, 16,000, and 16,700. The value 16,700 had been found for the dehydrogenation of succinic acid by methylene blue, a process known to be a function of the hydroxyl-ion concentration. Other processes catalyzed by hydroxyl ion show either $\mu = 16,700$ or $\mu = 11,500$. Many oxidations in which iron is known to be a catalyst show $\mu = 16,100$. In 1926, he collected the data on critical temperatures, i.e., temperatures at which the velocity constants of two reactions in the hypothetical con-

catenated series of reactions become approximately equal, so that this temperature marks the shifting point from one master reaction to another(4). More recently, with the finding that many reactions which are insensitive to cyanide show $\mu = 8000$ or $24,000$, there has been a tendency to regard critical thermal increments as of less significance than heretofore(9). At the present time, the finding of a constant μ value may be indicative of a single reaction controlling the concatenated series and may possibly suggest the type of catalyst involved. Further, the critical temperature seems to indicate a point at which the master reaction shifts.

The values for μ obtained in the dark, viz., 7200 , $16,900$, $23,600$, occur commonly. Whatever their significance may be, they are the same as have been found for the breathing rhythm of *Mustelus canis*, for the geotropic response of *Vicia faba*, and for many other biological reactions. It might be interesting to speculate upon the question of what reaction controls these diverse processes—perhaps an iron catalysis or an hydroxyl-ion catalysis as suggested by Rice(10). The one statement that can be made with reasonable certainty is that the master reaction is a rather simple process, and that the μ values indicate that it is one of a rather small group of reactions, thus narrowing the field of investigation and providing a useful clue.

The critical temperature which we have found at 19°C . has been found by Crozier(4) to be second in frequency only to the most common critical temperature: 15°C . The change in μ value from 7200 to $16,900$ that occurs at this temperature has been interpreted to indicate that the master reaction in the concatenated series of reactions has shifted. It is interesting to note that the values for μ are those which may be obtained in in vitro reactions, such as the enolization of glucose in slightly alkaline media.

The change in μ value to $23,600$ upon exposure at 46° for six hours may indicate the destruction of one of the catalytic systems in the cell; a similar change is described by Crozier and Stier(5) for the heart rate of *Limax*. And the practical

disappearance of oxygen consumption below 20°C. is further evidence favoring this hypothesis.

It becomes apparent that our results may be interpreted as showing that in the light we are not measuring a single, master reaction, since simple reactions show a constant value for μ , whereas our data for oxygen consumption in the light show that μ increases logarithmically as the temperature falls. This tends to confirm our hypothesis that during irradiation two concurrent reactions are proceeding which are differently affected by changes of temperature.

The final significance of this analysis, then, beyond that of presenting confirmatory evidence for the previous hypothesis, lies in indicating that despite the numerous differences which distinguish the biology of *S. lutea* from that of most cells, whether animal, vegetable, or bacterial, nevertheless the fundamental reactions of this cell's metabolism find many parallels throughout the animal and vegetable kingdom.

In conclusion, it seems interesting to describe briefly certain incidental observations on the effect of various environmental factors on *S. lutea*. We were interested in finding out what effect various salt concentrations and mixtures of various ions might have. First we measured the oxygen consumption of the cells in varying concentrations of NaCl, from zero to five molar. Figure 5 shows graphically the resultant data. There is a wide optimum range of salt concentration from about 0.1 N to about 1.8 N, but even the extremes of variation in osmotic pressure produced no measurable shrinkage or expansion of the cells, nor any marked cytolysis. After measuring the respiration of the cells for twenty-four hours, there was no significant alteration in viability.

Varying hydrogen-ion concentrations of the suspension medium was also tried, using a phosphate mixture over the entire range. The buffer was prepared by titrating M/15 KH_2PO_4 with either N/10 NaOH or N/10 HCl to the desired hydrogen-ion concentration. The buffer mixture was then used to dilute the cell suspension, 1.5 cc. of buffer being added to 0.5 cc. of cells. There is a rather narrow optimum band at

pH 5.8 to 6.2. The rate of respiration is slightly decreased on either side of the optimum, but does not fall off to zero until pH 1.4 on the acid side, or pH 10.7 on the alkaline side, as is shown in figure 6.

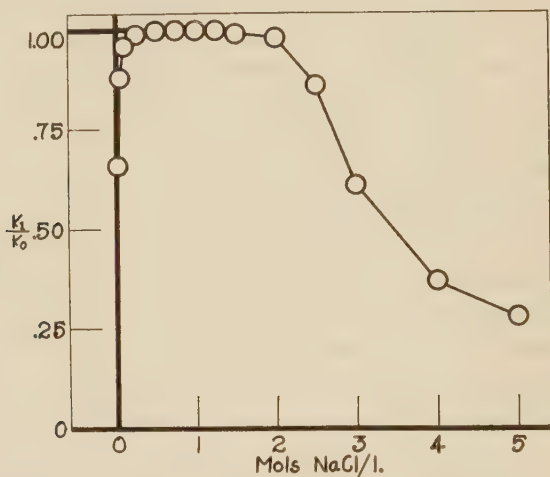


Fig. 5 $\frac{K_1}{K_0}$ refers to the velocity at the experimental salt concentration as compared with velocity at the optimum salt concentration.

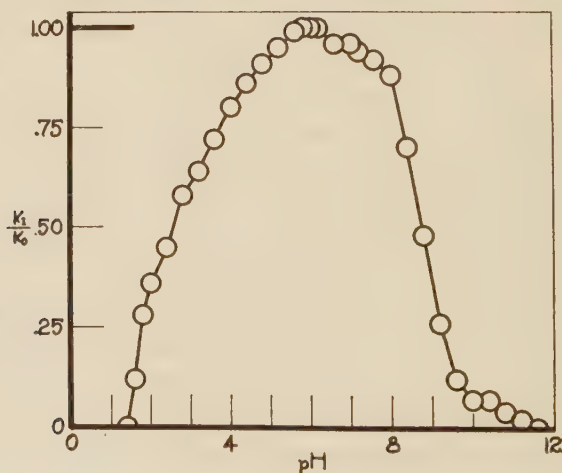


Fig. 6 $\frac{K_1}{K_0}$ refers to the velocity at the experimental hydrogen-ion concentration as compared with the velocity at the optimum hydrogen-ion concentration.

As has been reported by Gerard(7), we found practically no inhibition of respiration by cyanide or carbon monoxide. The cells do not grow in the absence of organic nitrogen, but remain viable for four to six days.

I wish to express my thanks to Dr. R. S. Lillie and to Dr. R. W. Gerard for their invaluable advice and assistance during this work and during preparation of this paper.

SUMMARY

The oxygen consumption of *S. lutea* in the dark may be described by the equation for a monomolecular reaction.

During irradiation, at temperatures below 30°, the rate of oxygen consumption is generally increased. At high temperature 35°, with low concentrations of substrate, there is a decrease in the rate of oxygen consumption.

An hypothesis to explain the differences in effect of irradiation has been advanced.

The inhibitor technique has not enabled us to isolate the master reaction.

A study of the effect of temperature shows commonly found values for the critical thermal increment: $\mu = 7200, 16,000, 24,000$ describing the oxygen consumption of *S. lutea* in the dark.

During irradiation, μ has a logarithmically increasing value as the temperature falls.

These results tend to confirm our previous hypotheses.

Some data on the viability of *S. lutea* under a wide range of conditions have been presented.

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THE KINETICS OF INTRACELLULAR CARBOHY- DRATE OXIDATION OF *SARCINA LUTEA*. II¹

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THREE FIGURES

(Received for publication December 2, 1932)

In an earlier paper(14) it was reported that the oxidation of glucose by *Sarcina lutea* seemed to obey the monomolecular law. Consequently, it became interesting to investigate in greater detail the intermediate stages in the oxidation of glucose by this organism.

It was soon found that oxygen is consumed if glucose is added to the fluid in which *S. lutea* had been suspended, after complete removal of the organisms by filtration. This indicated an outward diffusion of at least part of the intracellular enzymes concerned in the oxidation of sugar; and led to a study of oxygen consumption by the cell-free medium.

The problem of intracellular carbohydrate oxidation, particularly in muscle, has been reviewed comprehensively by Meyerhof(8) and recently very clearly by Dorothy Moyle Needham(9a). In general, there has been no reason to assume that the course of carbohydrate metabolism in bacteria is essentially different. In an extensive series of papers Neuberg(11) and his collaborators have worked out the intermediary carbohydrate oxidations of yeasts. They have found that glucose, after esterification with phosphoric acid, is converted into methyl glyoxal. This, in turn, is oxidized to pyruvic acid, with lactic acid as a possible intermediate product. Toeniessen and Brinkmann(16), indeed, suggest that lactic acid is never oxidized directly, but must first be re-

¹ This research was aided in part by a grant from the Rockefeller Foundation to The University of Chicago.

synthesized to carbohydrate. From a study of R. Q., Meyerhof and Boyland(9) showed that this was improbable. To the point of pyruvic acid formation, then, the course of events in muscle and in yeasts is similar. Barron(1), however, presents considerable evidence that in gonococcus glucose is oxidized to acetic acid without passing through methyl glyoxal as an intermediate. Indeed, the rate of oxidation of added methyl glyoxal is so slow as to indicate an almost complete absence of oxidase for it. Lactic acid is the invariable precursor of pyruvic acid in the gonococcus. According to the scheme suggested by Neuberg(10), pyruvic acid is further oxidized to acetic and formic acids. Barron(1) has found that pyruvic acid is oxidized by gonococcus to acetic acid and carbonic acid. Quastel(12) has suggested that pyruvic acid is probably resynthesized to lactic acid or more complex carbohydrate forms which are subsequently oxidized by another route. This author also suggested the possible conversion of pyruvic acid to alanine, based largely on the datum that in resting cells pyruvic acid accumulates, while in actively growing cultures it is very difficult to find any pyruvate at all, unless the fixation method of v. Grab(6) be used. This synthesis in vitro has been accomplished by Gerard(3).

EXPERIMENTAL DETAIL

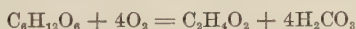
S. lutea was grown for 24 hours on 1 per cent peptone-agar slants. The cells were then washed from several slants into a centrifuge tube, shaken gently to provide uniform suspension and then centrifuged. The supernatant liquid was discarded. The cells were then resuspended in 5 cc. of a M/20 phosphate buffer mixture ($\text{pH} = 6.8$). Three hours later, the cells were filtered on a Berkefeld N porcelain filter. To 1 cc. of cell-free sterile filtrate (as tested by incubating in peptone broth for 48 hours) was added an equal volume of 0.1 N glucose or other substrate. The oxidation was then followed manometrically with the Warburg micro-respirometer. Controls for temperature, pressure, autoxidation of

substrate, and of cell-free suspension fluid were run simultaneously.

It should be stated explicitly that the oxygen consumption of any number of cells exceeds manyfold that of the cell-free suspension medium obtained from the same number of cells. However, when care is taken to adjust the glucose (or other substrate) and enzyme concentration to such a level that it is all oxidized in approximately 48 hours, by both the cell-free medium and by a relatively small number of cells, the data so obtained may be compared. This is justified by the well-known fact that, with a large excess of enzyme supplied from the beginning, further additions of enzyme, such as are undoubtedly elaborated by the living cells, have no appreciable effect on the rate of enzymatic conversion of substrate. At the temperature used in these experiments, $20^{\circ}\text{C.} \pm 0.01$, no appreciable destruction of enzyme occurs in the presence of its substrate. At a higher temperature, 37°C. , at least one of the enzymes is destroyed so rapidly that the oxidation process ends in 4 to 5 hours, when approximately only 50 per cent of the substrate has been oxidized.

THE DATA AND RESULTS

The data for glucose oxidation, presented in table 1, show that approximately two-thirds of the glucose is completely oxidized by the cell-free filtrate. However, on chemical analysis no glucose was found in the medium, which had a vinegar odor. Assuming, then, that each molecule is completely oxidized to acetic acid—



it is justifiable to attempt to fit a velocity constant for this reaction, if it takes place in an excess of oxygen. If the master reaction in the concatenated series of reactions involved in the oxidation of glucose lies between glucose and acetic acid, and if the enzymes and activators in the cell-free filtrate are the same as those in the cells, then the same constant should be found for the oxidation extracellularly as was

found intracellularly(14). After making the same assumptions, to facilitate treatment, it was indeed found that in the equation—

$$\frac{dy}{dt} = K(A - y), \quad K = 0.12 \text{ at } 20^{\circ}\text{C}.$$

This constant is identical with the one found for glucose oxidation by *S. lutea*, under the same conditions(14). This evidence indicates that there is present, in the cell-free filtrate, the same controlling mechanism and the same oxidizing enzymes, with the exception of the enzyme needed to convert acetic to formic acid.

It was possible, consequently, to compare the intermediate stages of glucose oxidation by providing a number of presumable intermediate products, viz., lactic acid, pyruvic acid (table 1); glycerol, ethylene glycol, ethyl and methyl alcohols were also used (table 2).

The results with lactic acid are very interesting. Oxidation of lactate by the cells is extremely rapid, nearly 16 per cent of added lactate being oxidized in the first quarter hour. However, the rate falls off more rapidly than can be explained by the law of mass action for a monomolecular or multimolecular equation. With the cell-free filtrate, oxidation goes to acetic acid as in the case of glucose, but in this case the reaction rate obeys the monomolecular formula and the equation can be written—

$$\frac{dO_2}{dt} = .62 (1 - O_2)$$

where $\frac{dO_2}{dt}$ is the rate of oxygen consumption, and O_2 is that fraction of the maximum oxygen consumed (100 per cent) at any time, t .

Except for the oxidation of fructose, which is essentially like that of glucose, the data for the oxidation of the other substances tried are not sufficiently regular and simple to admit of analysis of the kind previously found so useful. It is evident, however, that both pyruvic acid(5) and glycerol show an initial lag in oxidation, such as is encountered in autocatalytic reactions. In the case of pyruvate, the addition of either lactate or acetate reduced the initial lag mark-

TABLE 1
The oxygen consumption of the cell-free filtrate compared with that of *S. lutea* in various media

TIME IN HOURS	O ₂ OXYGEN CONSUMED BY 1 CC.						
	S. lutea in 1 cc. 0.1 M glucose	Cell-free filtrate in 1 cc. 0.1 M glucose	S. lutea in 1 cc. 0.1 M Na lactate	Cell-free filtrate in 1 cc. 0.1 N Na lactate	Cell-free filtrate in 1 cc. 0.1 N Na pyruvate	Cell-free filtrate in 1 cc. 0.1 N Na acetate	Cell-free filtrate in 1 cc. 0.05 N Na formate
	Av. of 28 expts.	Av. of 6 expts.	Av. of 9 expts.	Av. of 6 expts.	Av. of 3 expts.	Av. of 2 expts.	Av. of 2 expts.
1	0.44	0.29	1.28	0.31	0.00	0.00	0.09
1	0.85	0.56	1.89	0.59	0.03	0.00	0.15
1	1.26	0.83	2.64	0.81	0.04		0.21
1	1.65	1.10	3.28	1.02	0.06	0.00	
1½	2.41	1.60	4.33	1.39	0.10		0.26
2	3.11	2.07	5.10	1.58	0.22		
2½	3.78	2.51	5.67	1.76	0.97		
3	4.41	2.93	6.09	1.90	1.36		0.34
4	5.56	3.70	6.34	2.11	1.81		0.40
5	6.58	4.38	6.93	2.22	2.06		0.44
6	7.49	4.98	7.10	2.29	2.18	0.05	0.47
7	8.29	5.51	7.19	2.33	2.24		
8	9.01	5.99	7.23	2.36			
9	9.64	6.40	7.27	2.37		0.08	0.54
10	10.21	6.78	7.28	2.38	2.29		
11	10.73	7.13	7.29	2.39			
12	11.17	7.42	7.29	2.39	2.34	0.06	0.57
15	12.19	8.10	7.30	2.40	2.36	0.08	
18	12.92	8.58					
20	13.27	8.82					
24	13.81	9.18				0.09	0.57
48	14.56	9.67				0.10	

TABLE 2
A continuation of table 1

TIME IN HOURS	CC. OXYGEN CONSUMED BY 1 CC. (SINGLE EXPERIMENTS)					
	S. lutea in 1 cc. 0.1 N fructose	Cell-free filtrate in 1 cc. 0.1 N fructose	Cell-free filtrate in 1 cc. 0.1 N glycerol	Cell-free filtrate in 1 cc. 0.1 N ethylene glycol	Cell-free filtrate in 0.1 cc. 0.1 N ethyl alcohol	Cell-free filtrate in 1 cc. 0.1 N methyl alcohol
$\frac{1}{4}$	0.42	0.31	0.09	0.06	0.21	0.37
$\frac{1}{2}$	0.85	0.58	0.18	0.12	0.37	0.49
$\frac{3}{4}$	1.21	0.79	0.25	0.18	0.52	0.59
1	1.59	1.03	0.30	0.23	0.66	0.65
$1\frac{1}{2}$	2.18	1.50	0.36	0.31	0.84	0.73
2	3.00	2.00	0.50	0.40	0.97	0.81
$2\frac{1}{2}$	3.53	2.48	1.27	0.46	1.10	0.90
3	4.16	2.89	1.70	0.51	1.15	0.96
4	5.05	3.62	2.21	0.59	1.17	1.01
5	5.93	4.24	2.63	0.65	1.19	1.05
6	6.62	4.86	2.78	0.69	1.20	1.08
7	7.28	5.48	2.85			
8	8.57	6.10	3.11			
9	9.18	6.61	3.32			
10	9.80	7.18				
11	10.22	7.53				
12	10.68	7.92	3.54			
15	12.11	8.39	3.64			
18	13.14	8.80				
24	13.75	9.17	3.69			
48	14.43	9.36				

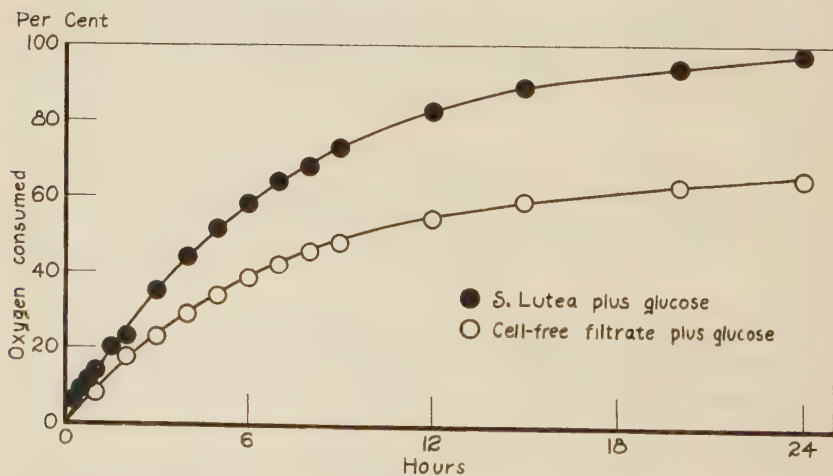


Fig. 1 Comparison of oxygen consumption by *S. lutea* and the cell-free filtrate. In absolute value (cc. of O_2 consumed) the cell-free filtrate uses only two-thirds as much oxygen as the cells. If, however, this value were also set at 100 per cent, the two curves would be superimposed.

edly, as can be seen in figure 2. Lactate also reduced the lag of glycerol oxidation, but not very markedly. Acetic acid is not noticeably oxidized by the cell-free filtrate, although its oxidation by a cell suspension is extremely rapid (5). Formic acid and methyl alcohol are both rapidly oxidized to carbonic acid.

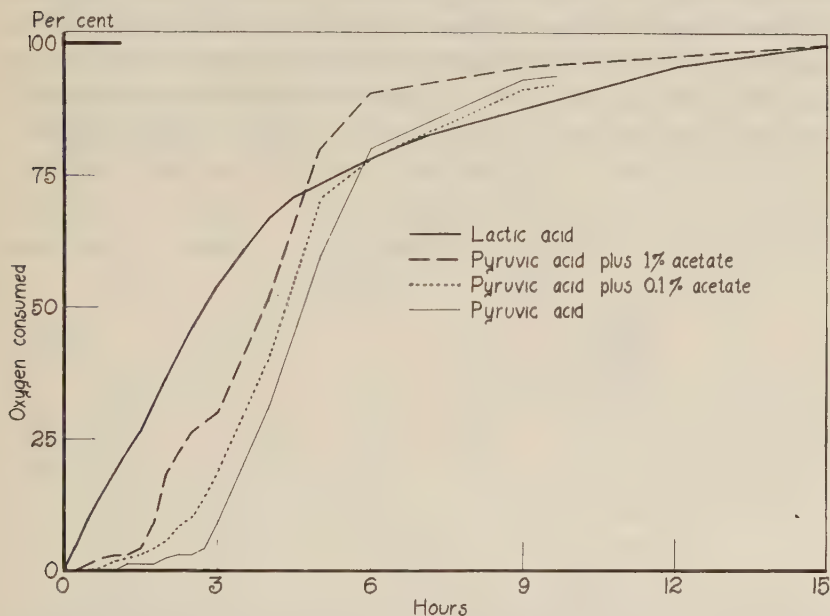


Fig. 2 Comparison of oxidation of lactic and pyruvic acids. A reduction in the lag period occurs when either lactic or acetic acid is added to pyruvic acid, after making the necessary reduction for the oxygen consumption by these added substances.

DISCUSSION

Inasmuch as the k for glucose is smaller than that of any intermediate product we have investigated, it can be said that this investigation has failed to give direct evidence for the position of the master reaction. From the evidence of Schäffer and Friedemann(15) for muscle and of Neuberg(11) for yeasts, it may be considered most likely that the master reaction in the intracellular glucose oxidation involves the

activation of glucose. Further evidence for this view may be derived from the fact that enolizations of glucose have approximately the same thermal increments(13) as are shown by *S. lutea* for the oxidation of glucose(14).

Further experiments to test this hypothesis have been performed. It can be seen (table 3) that the optimum pH for the oxidation of lactic and pyruvic acids by the cell-free filtrate is on the acid side of neutrality. Cell-free filtrate was added rapidly to a faintly alkaline ($\text{pH}=7.2$) glucose solution and the oxygen consumption was followed manometrically, as before. If enolization, which is markedly accelerated in alkaline solution, is the controlling reaction, then

TABLE 3

The range of pH and optimum pH at which glucose and its presumable intermediates may be oxidized by S. lutea

	GLUCOSE	FRUCTOSE	GLUCOSE (AFTER ANAEROBIOSIS)	LACTIC ACID	PYRUVIC ACID	ACETIC ACID	FORMIC ACID	GLYCEROL	ETHYLENE GLYCOL	ETHYL ALCOHOL	METHYL
Range	1.8-8.6	2.0-9.6	3.5-8.8	1.8-8.5	4.1-7.7	2.0-10.0	2.2-10.0	4.4-9.5	4.4-10.0	3.1-9.8	2.3-
Optimum	7.4	7.4	6.6	6.3	6.1	6.8	6.5	5.9	5.9	6.2	6.

this treatment should markedly accelerate the oxidation of glucose. This was indeed found to be true, when the glucose solution was made somewhat more alkaline ($\text{pH}=7.6$) or when the temperature was raised (to $37^{\circ}\text{C}.$).

Another experiment to test this hypothesis was suggested by Dr. R. W. Gerard. If either *Sarcinae* or the cell-free filtrate were allowed to act on a glucose solution anaerobically, then an equilibrium between inactive and active glucose should be established after a certain period of time. Keeping the mixture anaerobic for a longer period should not alter this equilibrium. Upon admitting oxygen, therefore, the rate of oxygen consumption should be markedly increased, although the total oxygen consumed should remain constant. This experiment was performed, using the nitrogen technique

of Gerard, for anaerobiosis(4); the results provide a striking confirmation for the hypothesis, as can be seen in figure 3. Finally, the results of Brönsted and Guggenheim(2), who followed enolization of glucose polarimetrically, support this, for they find a value of $k=0.15$ at 25° at pH 6.96.

Thus, by a process of elimination, the activation of glucose has been implicated as being the slowest in the concatenated series of reactions of glucose oxidation. The direct evidence for this, viz., the actual determination of k for this reaction alone, is at present wanting, since no method of stopping

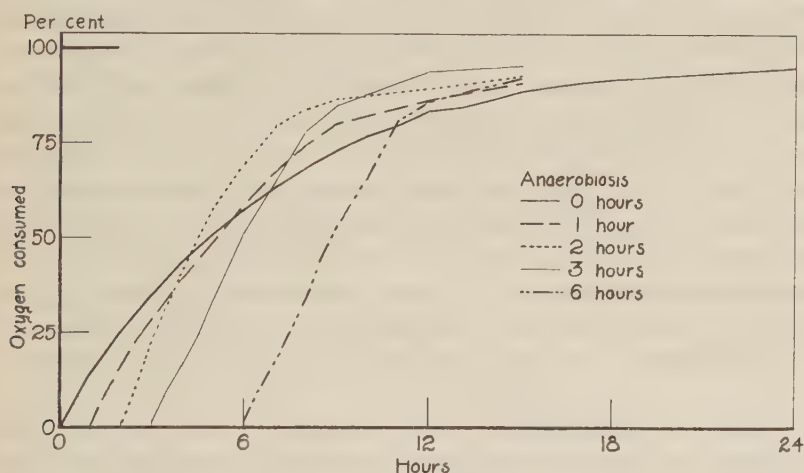


Fig. 3 The effect of anaerobiosis for different lengths of time on the initial rate of oxidation of glucose. The final oxygen-consumed level is equal in all cases.

the reaction at the point where α or β glucose is converted into γ glucose is known.

By simultaneous comparison of the k values obtained in the oxidation of each of the intermediate products of glucose oxidation in cell-free enzyme mixtures with the k values obtained by supplying these same products to the cells, a method for studying the *in vivo* carbohydrate metabolism is now available. This method may well be supplemented by using the 'inhibitor' techniques, i.e., by introducing into an enzyme mixture (whether intra- or extracellular) an enzyme inhibitor, viz., iodoacetic acid which inhibits lactic-acid formation(7) and resynthesis(9).

For *S. lutea*, therefore, the following initial reactions of intracellular carbohydrate oxidation are proposed.

1. Glucose is activated.

2. Active glucose is converted into lactic acid or pyruvic acid or some two-carbon substance which is further oxidized.

The first step in this proposed scheme is the master reaction. It is monomolecular under the conditions of this experiment with a velocity constant $K = 0.12$ at 20°C . It is catalyzed by hydroxyl ions. The second step may involve the intermediate formation of possibly three compounds: hexose phosphate, glyceric aldehyde, methyl glyoxal. The data dealing with these substances are extremely confusing. The evidence in these experiments, as in those of Barron, indicates that methyl glyoxal is not involved. Also, while the intermediate formation of lactic acid is not inconsistent with the velocity data, the only way to bring into accord the utilization of 4O_2 per mol of glucose would be to postulate the formation of but one molecule of lactic acid from each molecule of glucose. It is quite possible that both of two paths are employed, one involving pyruvic acid, the other splitting lactic into acetic and formic acids more directly.

I wish to acknowledge the invaluable suggestions and criticism of Dr. R. S. Lillie and of Dr. R. W. Gerard. I wish to thank Mr. M. Taitel for his help in drawing the charts.

SUMMARY

The cell-free filtrate of *S. lutea* was found to contain the enzymes necessary for the oxidation of sugar to acetic acid. The kinetics of this process were studied and compared with the kinetics of the process when the cells were used. No essential differences were found.

The oxidation of a number of presumably intermediate products was also studied. By a process of elimination, the master (or slowest) reaction was found to be the activation of glucose.

A scheme of intracellular carbohydrate oxidation is proposed.

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